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CUTTING EDGE

Induction of Tissue-Specific Autoimmune Prostatitis with Prostatic Acid Phosphatase Immunization

Implications for Immunotherapy of Prostate Cancer[1]

Lawrence Fong,[2]* Curtis L. Ruegg,† Dirk Brockstedt,* Edgar G. Engleman,* and Reiner Laus†

Prostatic acid phosphatase (PAP) is uniquely expressed in prostatic tissue and prostate cancer. In this study, the immunogenicity of PAP was investigated in a male rat model. We show that immunization with recombinant rat or human PAP in CFA leads to a significant Ab response, but does not generate CTL or result in autoimmune prostatitis. In contrast, immunization with recombinant vaccinia expressing human PAP, but not rat PAP, generates a CTL response and tissue-specific prostatitis in the absence of detectable PAP-specific Abs. These findings suggest that a cellular immune response to PAP, rather than Abs, mediates destructive autoimmune prostatitis. Thus, xenogeneic forms of PAP are a new tool for the induction of prostate-specific immunity and may prove useful for the immunotherapy of prostate cancer. ***The Journal of Immunology,*** **1997, 159: 3113–3117.**

Diseases of the prostate are common among aging men. Prostatic hyperplasia and hypertrophy are almost universal in older men, and prostate cancer has become the most common malignancy and the second leading cause of cancer-related death in men (1). The treatment of localized prostate cancer has improved in recent years. The treatment of disseminated disease relies on hormonal therapy with systemic estrogens or luteinizing hormone-releasing hormone analogues or on androgen ablation through bilateral orchiectomy, often in combination with antiandrogens. However, in patients with hormone refractory disease, these treatments are no longer effective, and other treatments are palliative and do not prolong survival (1, 2). Relapses of hormonally treated prostate cancer almost always are due to the growth of androgen-independent tumor cells for which no effective treatment is available (2, 3).

In these experiments, we investigate whether an immune response can be generated to proteins selectively expressed in the prostate, and if so, whether such responses lead to the destruction of prostate tissue including prostate tumor cells. Prostatic tissues and prostate cancer express several tissue-specific Ags, including prostatic acid phosphatase (PAP),[3] prostate-specific Ag, and prostate-specific membrane Ag (PSMA) (4–6). However, PAP exhibits no more than 50% amino acid homology to any known protein, while prostate-specific Ag has 78 and 57% homology to human glandular and pancreatic/renal kallikrein, respectively. Moreover, PSMA appears to be expressed in nonprostatic tissues, including brain, salivary gland, and small intestine, when examined by immunohistochemistry (7). PAP, therefore, appears to be a more selective target for immunotherapy. The gene for human PAP (hPAP) contains a 1065-nucleotide coding region that gives rise to a 354-amino acid polypeptide with an estimated molecular mass of 41 kDa. mAbs with specificity for PAP stain only normal prostate and prostate cancer cells, and the use of PAP cDNA to probe Northern blots from a variety of tissues reveals PAP RNA only in benign and malignant prostate cells (8, 9). The rat homologue of PAP (rPAP) shares 75% protein homology with hPAP as well as a similar pattern of expression limited to prostatic tissues (10).

In this study, the ability of PAP to serve as an immunogen was explored in Copenhagen (COP) rats. The Dunning tumor lines, among the most extensively studied prostate carcinoma cell lines, were derived from a tumor isolated from an aged COP rat. Several different Dunning tumor sublines express rPAP, making these sublines useful as immunologic targets for PAP-specific CTL (11). By examining the endogenous prostate gland for evidence of inflammation, biologically significant immune responses toward this specific tissue were assessed.

Materials and Methods

PAP constructs

The cDNAs encoding rPAP and hPAP were cloned into the pBacPAK8 baculovirus recombination vector (Clontech, Palo Alto, CA) or into a vaccinia virus (vv) recombination vector. Rat PAP cDNA was amplified from first-strand cDNA made from mRNA isolated from rat prostate (Harlan,

*Stanford University School of Medicine, Stanford, CA 94305; and †Activated Cell Therapy, Inc., Mountain View, CA 94043

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[2] Address correspondence and reprint requests to Dr. Lawrence Fong, Stanford Blood Center, 800 Welch Road, Palo Alto, CA 94304.

[3] Abbreviations used in this paper: PAP, prostatic acid phosphatase; PSMA, prostate-specific membrane antigen; hPAP, human prostatic acid phosphatase; rPAP, rat prostatic acid phosphatase; COP, Copenhagen; vv, vaccinia virus; vv-PAP, vaccinia virus expressing prostatic acid phosphatase; wt, wild type.

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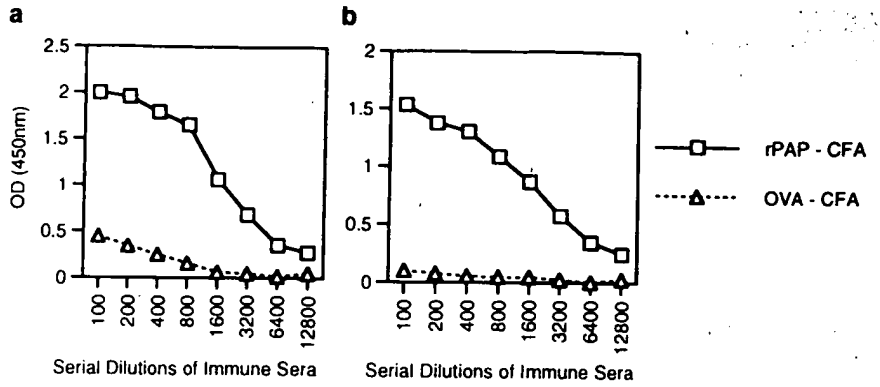

FIGURE 1. Anti-PAP Abs can be generated by immunizing rats with rPAP in CFA. Immune sera from rats immunized with rPAP in CFA or with OVA in CFA were assessed by ELISA for Ab responses against rPAP (*a*) or hPAP (*b*). Each line represents the average OD of sera from three animals per group.

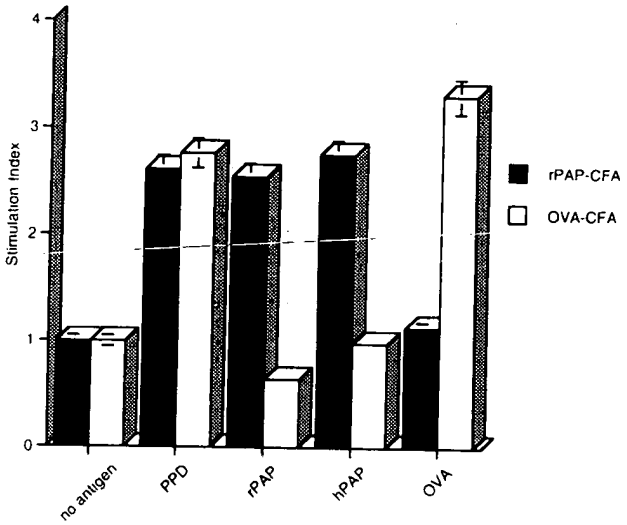

FIGURE 2. Lymphocytes from rats immunized with rPAP in CFA proliferate in response to rPAP and hPAP. Lymphocytes from draining lymph nodes were pooled from three rats per group immunized with either rPAP or OVA in CFA and were cultured at 500,000 cells/well in 96-well flat-bottom plates with no Ag, PPD at 2.5 μg/ml, or rPAP or hPAP Ags at 10 μg/ml. The proliferation of triplicate cultures was assessed after 4 days of culture by 18-h [^{3}H]thymidine incorporation (mean ± SD). PPD represents a positive control recall Ag for CFA. OVA represents an irrelevant Ag.

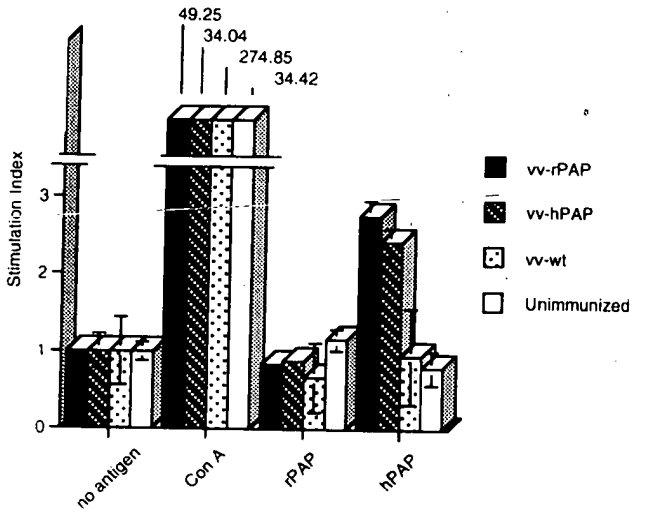

FIGURE 3. Splenocytes from rats immunized with recombinant vv proliferate in response to hPAP. Splenocytes pooled from three rats per group immunized with vv-rPAP, vv-hPAP, or vv-wt or unimmunized were cultured at 500,000 cells/well in 96-well flat-bottom plates with no Ag or with Con A at 2.5 μg/ml or PAP Ags at 10 μg/ml. Proliferation was assessed in triplicate wells by 18-h [^{3}H]thymidine incorporation after 4 days of culture (mean ± SD). Con A represents a positive control to assess the viability of the splenocytes.

Indianapolis, IN) using primers that amplify the fragment containing nucleotides 15 to 1177 (GenBank accession no. M32397). The cDNA encoding hPAP was amplified by PCR from first-strand cDNA made from mRNA isolated from the human prostate carcinoma cell line LNCaP (American Type Culture Collection, Rockville, MD; CRL 1740) using primers that delineate the fragment containing nucleotides 1 to 1175 (GenBank accession no. M34840). They were used to generate recombinant baculoviruses and vv by homologous recombination.

To facilitate purification, recombinant rPAP was expressed as a His^6 fusion protein. Insect SF21 cells were infected with recombinant baculovirus, and rPAP was purified from culture supernatants with a Ni-NTA column (Qiagen, Hilden, Germany) to >70% purity by SDS-PAGE. Human PAP used for proliferation assays and ELISA was purified from human semen (Biodesigns, Kennebunk, ME). Vaccinia viruses were grown in COS-7 cells.

Proliferation assays

Harvested lymph nodes or spleen were mechanically teased into a single-cell suspension. RBC were lysed with ammonium chloride. The remaining cells were cultured at 500,000 cells/well in 96-well flat-bottom plates (Costar, Cambridge, MA) in Iscove's modified Dulbecco's medium supplemented with 0.5% autologous rat serum, antibiotics, and 2-ME. Corresponding Ags were also added at concentrations of 5 to 20 μg/ml. PPD (Connaught, Swiftwater, PA) and Con A (Sigma Chemical Co., St. Louis, MO) were used as positive controls. Proliferation was assessed on the basis of 18-h [^{3}H]thymidine incorporation after 4 days of culture as measured using a Microbeta counter (Wallac, Turku, Finland). The results are expressed as stimulation indexes representing counts per minute relative to baseline counts without Ag.

ELISA

Ninety-six-well Immulon-4 plates (Dynatech, Chantilly, VA) were coated overnight at 4°C with rPAP, hPAP, or OVA (Sigma Chemical Co.), blocked with 3% BSA in 50 mM Tris-buffered saline and 0.05% Tween-20, and washed with 50 mM Tris-buffered saline and 0.05% Tween-20. Rat serum was diluted in PBS, added to wells, and incubated for 1 h at room temperature. Plates were then washed, incubated with goat anti-rat horseradish peroxidase-labeled Ab for an additional hour at room temperature, developed with tetramethylbenzidine (Zymed, South San Francisco, CA), halted with 1 N HCl, and read at 450 nm with the Titer-Tek Multiscan plate reader (Titer-Tek, Helsinki, Finland).

Cytotoxicity assays

Pooled splenocytes were stimulated in vitro with vv-rPAP at a multiplicity of infection of 0.01 or with the irradiated (30,000 rad) Dunning tumor line Mat-LyLu at a 10:1 ratio of splenocytes to tumor cells. After 7 days of culture, splenocytes were assessed for cytotoxicity against the Dunning tumor cell lines AT1 (PAP negative) and AT3 (PAP positive) by a standard 4-h chromium release assay. Briefly, target cells were incubated with 0.25

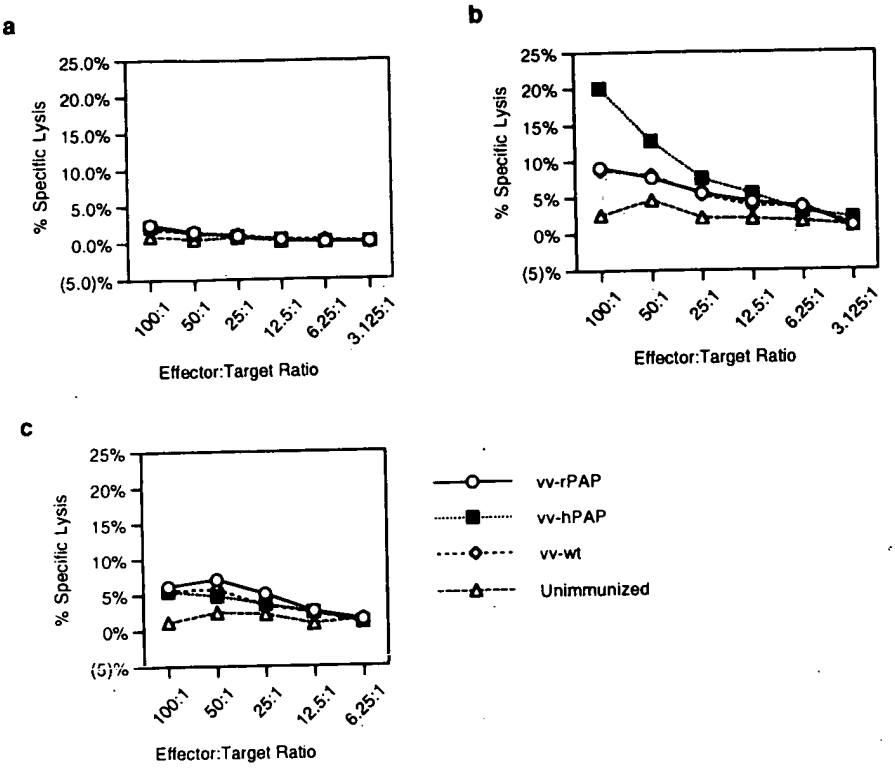

FIGURE 4. Immunization with hPAP expressing vv induces CTL. Cellular cytotoxicity in vv-immunized rats against *a*, rPAP negative Dunning cell line AT-1; *b*, rPAP positive Dunning cell line AT-3; or *c*, AT-3 preincubated with anti-MHC class I Ab. Pooled splenocytes from unimmunized rats or rats immunized with vv-hPAP, vv-rPAP, or vv-wt were restimulated in vitro for 1 wk with the irradiated Dunning tumor line Mat-LyLu at a 10:1 ratio of splenocytes to tumor cells. Thereafter, the cultured cells were assessed for cytotoxicity by a standard 4-h ^{51}Cr release assay in triplicate wells (mean ± SD). The results represent pooled data from three rats per group. SDs for all data points were ≤1%. Spontaneous ^{51}Cr release in the absence of effectors was always <10% of maximal release. The data shown are from a single experiment, which is representative of three experiments from three separate cohorts with similar results.

Table I. *Frequency of prostatitis in vv-immunized rats*[a]

	No Inflammation	Interstitial Inflammation	Destructive Inflammation
vv-rPAP	4/5	1/5	0/5
vv-hPAP	0/5	1/5	4/5
vv-wt	4/5	1/5	0/5
Unimmunized	3/3	0/3	0/3

[a] Vaccinia-immunized rats were sacrificed between 1 and 3 mo after i.v. immunization with 2×10^7 PFU of vv-rPAP, vv-hPAP, or vv-wt. Age-matched unimmunized rats served as an additional control group. Sections (6 μm) were stained with hematoxylin-eosin from multiple tissues, including the prostate, and scored for inflammation in a blinded fashion. Interstitial Inflammation represents presence of inflammatory cells in the stromal tissues only; Destructive Inflammation represents presence of inflammatory cells infiltrating the glandular epithelium and distorting glandular architecture. Numbers represent animals/group.

mCi ^{51}Cr for 2 h and then washed in cold medium. Target cells were cocultured at 10,000 cells/well in 96-well U-bottom plates (Costar) with cultured splenocytes at the indicated E:T ratios in triplicate wells. After 4-h culture, supernatants were harvested, mixed with Optiphase scintillation fluid (Wallac), and counted in a Microbeta counter. The percent specific lysis was calculated by the formula: 100% × (experimental release − spontaneous release)/(maximum release − spontaneous release). Maximum release was determined by lysis of target cells in PBS containing 0.5% Triton X-100 (Sigma Chemical Co.). In blocking studies, anti-rat MHC class I (RT1A) and class II (RT1B) Abs (PharMingen, San Diego, CA) were preincubated with labeled targets at 25 μg/ml for 30 min before the ^{51}Cr release assay. Both Abs have previously been demonstrated to block MHC class I- and class II-mediated cellular responses.

Histology

The lower genito-urinary tracts, including ventral and dorsal prostate lobes, coagulating glands, and bladder, as well as normal tissues, including lung, liver, kidney, heart, and intestine, were removed from the animals and fixed in 10% formaldehyde. Six-micron sections were made from paraffin-embedded tissues at 3 to 5-mm intervals and stained with hematoxylin-eosin.

Results

Immunogenicity of rPAP in CFA

To determine whether an immune response could be mounted against PAP, COP rats were immunized with recombinant rPAP or hPAP in CFA. Rat and human PAP were produced and purified in a recombinant baculovirus system. Six- to eight-week-old COP male rats were immunized with 100 μg of rPAP or hPAP in CFA s.c. at the base of the tail. Animals were subsequently boosted twice with the same Ag in IFA s.c. at 2-wk intervals. The animals were killed 4 wk following the final boost. Sera, regional lymph nodes, and splenocytes were assessed for Ab, proliferation, and CTL responses, respectively. Prostate glands were also assessed by histology for evidence of inflammation.

As shown in Figure 1, rats immunized with rPAP in CFA developed significant Ab titers to rPAP that also cross-reacted with hPAP (Fig. 1), indicating that Abs could be generated to this self Ag. Lymphocytes from draining lymph nodes proliferated in response to both rPAP and hPAP (Fig. 2). Similar Ab and proliferative responses were generated after immunization with hPAP using this protocol (data not shown). However, immunization with either rat or human PAP in CFA did not generate CTL or evidence of prostatitis (data not shown).

Immunogenicity of recombinant vv expressing PAP

We next examined the immunogenicity of vv constructs expressing PAP. Six- to eight-week-old COP male rats were immunized i.v. with 2×10^7 plaque-forming units of recombinant vv expressing rPAP (vv-rPAP), hPAP (vv-hPAP), or the parental WR strain (vv-wt). These animals were killed 1 to 3 mo later and assessed for Ab, proliferation, and CTL responses.

In contrast to immunization with rPAP or hPAP in CFA, immunization with either vv-rPAP or vv-hPAP elicited no Abs against rat or human PAP (data not shown). On the other hand, splenocytes from rats immunized with vv-rPAP or vv-hPAP proliferated in response to hPAP protein, but not to rPAP, suggesting

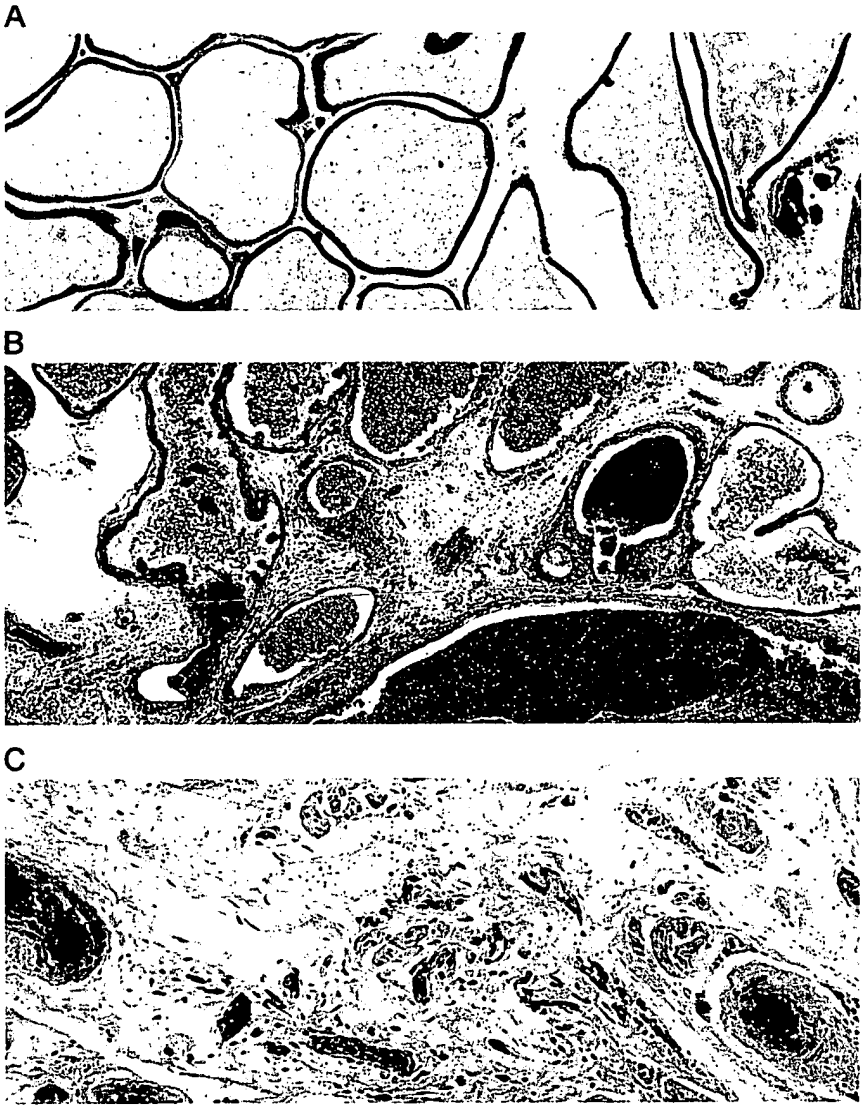

FIGURE 5. Immunization with hPAP expressing vv induces prostatitis. Vaccinia-immunized rats were killed between 1 and 3 mo after i.v. immunization with 2×10^7 plaque-forming units of vv-rPAP, vv-hPAP, or vv-wt. Their prostates were removed, fixed in 10% formalin, and embedded in paraffin. Sections stained with hematoxylin-eosin from rats immunized with vv-rPAP (*A*; magnification, ×40) and vv-hPAP (*B*; magnification, ×40) were examined. One rat immunized with vv-hPAP had only fibrotic tissue remaining in the prostate (*C*; magnification, ×40).

that vv immunization generates a T cell-mediated proliferative response to cross-reactive epitopes (Fig. 3). Moreover, CTL activity against the rPAP-expressing tumor cell line, AT-3, was seen only in rats immunized with vv-hPAP, not in vv-rPAP, vv-wt, or unimmunized rats (Fig. 4*b*). No cytotoxicity was demonstrated against the rPAP-negative cell line, AT-1 (Fig. 4*a*). When the AT-3 line was preincubated with anti-MHC class I Ab (Fig. 4*c*), lytic activity was blocked, suggesting an Ag-specific, MHC class I-restricted response. Preincubation with anti-MHC class II Ab (RT1B) did not block lytic activity (data not shown).

When histologic sections were made of various tissues from vv-immunized rats, prostate-specific inflammation involving the glandular epithelium was found only in rats immunized with vv-hPAP, not in those immunized with vv-rPAP or vv-wt (Table I). The prostates in four of five rats receiving vv-hPAP had evidence of cellular invasion of the glandular epithelium associated with disruption of the secretory architecture (Fig. 5*B*). Moreover, one of these rats developed hematuria and was found to have a fibrotic prostate with only rare distorted glandular elements, consistent with near-total ablation of the prostate (Fig. 5*C*). Other tissues, including coagulating glands, lung, liver, kidney, brain, and gastrointestinal mucosa, showed no evidence of inflammation.

Discussion

While the prostate is an important component of male sexual physiology, it clearly is a nonessential organ given the paucity of systemic problems associated with therapeutic removal or ablation of prostate glands. With the high prevalence of prostatic disease in aging men, specific destruction of prostate-derived tissues may therefore be desirable, especially in patients with metastatic prostate cancer. In fact, immunotherapy of prostate cancer has been attempted in a phase I clinical trial using PSMA peptide-pulsed dendritic cells to treat prostate cancer (12).

The current experiments demonstrate the feasibility of generating an immune response against the prostate and open the possibility of exploiting this approach for therapy. The generation of an immune response to PAP in a syngeneic animal model shows that preexisting tolerance to this self antigen can be broken. PAP was immunogenic when delivered either as whole protein mixed with adjuvant or as a recombinant vv encoding whole protein. While immunization with rat or human PAP in CFA led to Ab and proliferative responses, no evidence of prostate inflammation could be demonstrated. Abs to PAP, therefore, do not appear to play a role in pathologic autoimmunity in this animal model. In contrast, administration of recombinant vv containing genes encoding rPAP or

hPAP did not generate measurable Ab responses to rat or human PAP. Proliferative responses to hPAP could be detected in vv-rPAP- and vv-hPAP-immunized rats, suggesting that T lymphocytes recognizing cross-reactive epitopes to hPAP were expanded. However, CTL activity could only be detected in rats immunized with vv-hPAP. Most likely, this lytic activity was mediated by T lymphocytes recognizing immunodominant MHC class I epitopes derived from hPAP that cross-react with rPAP. This pattern has been previously described with induction of tolerance to self proteins in transgenic models (13).

Prostate-specific inflammation was seen only in rats that developed PAP-specific CTL after receiving vv-hPAP. Although experimental autoimmune prostatitis has been described in rodents, these experiments typically use prostatic homogenates rather than a specific Ag and are typically self-limited and not destructive (14–16). The importance of cell-mediated immunity in certain autoimmune as well as tumor immunology models has been well described (17–19). Furthermore, the role of xenoantigens in the induction of autoimmunity has been demonstrated in several models, including experimental autoimmune encephalomyelitis and thyroiditis (20–22). Xenoantigens may, therefore, be useful for purposes of breaking tolerance to heterologous self Ags. In this model, sensitization of the immune system to xenoantigen leads to the generation of T lymphocytes that can cross-react with self PAP or, alternatively, to the phenomenon of epitope spreading that results in tissue-specific inflammation and destruction of the prostate gland. Employing xenogeneic homologues of self Ags as molecular mimics may, therefore, constitute an effective means of immunizing against self Ags preferentially expressed on tumors.

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